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The cryoprotective effects of erythritol on frozen-thawed ram sperm

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ABSTRACT

This study was conducted to evaluate the effect of replacing glycerol with erythritol on cryopreservation of ram spermatozoa. Semen samples (n=24) were collected from four rams in six times. In each session, the collected ejaculates (n=4) were pooled and split into 12 equal parts. The amount of 0.032 M glycerol (G32E0, equal to 3% glycerol), 0.016 M glycerol and 0.016 M erythritol (G16E16), 0.008 M glycerol and 0.024 M erythritol (G8E24), 0.032 M erythritol (G0E32), 0.054 M glycerol (G54E0, equal to 5% glycerol), 0.027 M glycerol and 0.027 M erythritol (G27E27), 0.013 M glycerol and 0.041 M erythritol (G13E41), 0.054 M erythritol (G0E54), 0.076 M glycerol (G76E0, equal to 7% glycerol), 0.038 M glycerol and 0.038 M erythritol (G38E38), 0.019 M glycerol and 0.057 M erythritol (G19E57) and 0.076 M erythritol (G0E76) were added. The diluted samples were frozen using standard protocol. After thawing, the samples were incubated at 37°C for 6 h. Results showed that progressive sperm motility and acrosome integrity were higher in G13E41 (18.85 % and 27.41 %, respectively) than treatments that contained only glycerol at 6 h ($p < 0.05$). At the level of 0.032 and 0.054 M cryoprotectant, the highest of total sperm motility was observed in G8E24 (19.16 %) and G13E41 (18.85 %) at 6 h, respectively ($p < 0.05$). Therefore, the quality of frozen-thawed ram spermatozoa can be improved by using the mixture of 0.013 M glycerol plus 0.041 M erythritol or 0.008 M glycerol plus 0.024 M erythritol.

Keywords

Cryopreservation, Polyol, Glycerol, Ram sperm

Abbreviations

G32E0: group treated with 0.032 M glycerol
G16E16: group treated with 0.016 M glycerol + 0.016 M erythritol
G8E24: group treated with 0.008 M glycerol + 0.024 M erythritol
G0E32: group treated with 0.032 M erythritol
G54E0: group treated with 0.054 M glycerol
G27E27: group treated with 0.027 M glycerol + 0.027 M erythritol
G13E41: group treated with 0.013 M glycerol + 0.041 M erythritol
G0E54: group treated with 0.054 M erythritol
G76E0: group treated with 0.076 M glycerol
G38E38: group treated with 0.038 M glycerol + 0.038 M erythritol
G19E57: group treated with 0.019 M glycerol + 0.057 M erythritol
G0E76: group treated with 0.076 M erythritol

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Introduction

Cryopreservation allows for the long-term storage of spermatozoa, which is highly advantageous in a number of reproductive fields. Also, it is vital for animal genetic maintenance and propagation. The semen of ram can be frozen for long-term storage, but when the fertility rate is acceptable, frozen-thawed spermatozoa should be inseminated using intrauterine insemination via the laparoscopic method [1]. Yet, sheep breeders would rather use the vaginal or intracervical artificial insemination, as simple and cheap methods, than laparoscopic intrauterine artificial insemination [2]. Therefore, it seems necessary to improve the freezing methods of ram spermatozoa.

Glycerol has been used commonly as a cryoprotectant in the freezing of diluted ram semen [3]. However, there is evidence that the presence of glycerol in the diluted semen depresses fertility in ovine [4]. Moreover, glycerol accelerates the acrosome reaction of ram spermatozoa [5, 6]. The negative effects of glycerol have led to studies on the replacement of glycerol with other cryoprotective agents [7, 8]. However, these studies did not come with satisfactory results.

Erythritol is a four carbon sugar alcohol and this polyol can be used as a cryoprotective agent [9, 10]. In the mammalian cell, erythritol is not metabolized to toxic metabolites, but, glycerol can produce toxic agents [11]. The aim of the present study was to evaluate the effect of replacing glycerol with erythritol on the process of cryopreservation of ram spermatozoa.

Results

Results showed that erythritol did not affect the membrane integrity, viability, total and progressive sperm motility, curvilinear velocity, average path velocity and straight line velocity at 0 and 3 h after thawing ($p > 0.05$).

At the level of 0.032 and 0.054 M cryoprotectant, the highest of total sperm motility was observed in G8E24 (29.64 % ± 1.41) and G13E41 (24.74 % ± 1.20) at 6 h, respectively (Fig 1, $p < 0.05$). There was no difference between treatment content of 0.076 M cryoprotectant on the total sperm motility at 6 h ($p > 0.05$). Progressive sperm motility was higher in G8E24 (19.16 % ± 1.18) and G13E41 (18.85 % ± 1.42) than G32E0 (11.1 % ± 0.56), G3E1 (11.92 % ± 1.09), G54E0 (11.04 % ± 0.44), G27E27 (11.09 % ± 0.29), G76E0 (12.80 % ± 1.68) and G0E76 (11.44 % ± 0.50) at 6 h (Fig 2, $p < 0.05$). Curvilinear velocity (Fig 3), average path velocity (Fig 4) and straight line velocity (Fig 5) was higher in G8E24 (50.65 ± 2.41, 31.07 ± 1.48, 30.22 ± 1.44, respectively) than treatments with content of 0.032 and 0.076 M cryprotectant at 6 h after thawing ($p < 0.05$)

There was no difference among G8E24 (22.45 % ± 0.58), G27E27 (22.92 % ± 1.96), G13E41 (23.42 % ±0.76) and G19E57 (22.57 % ± 0.39) on the sperm viability at 6 h (Fig 6, $p > 0.05$). Sperm viability was higher in these treatments than G32E0 (17.47 % ± 0.47) and G76E0 (17.22 % ± 0.74) at 6 h ($p < 0.05$).

Membrane integrity was higher in G13E41 (23.43 % ± 1.04) than G32E0 (18.08 % ± 0.83) at 6 h (Fig 7, $p < 0.05$) and there was no difference among other treatments ($p < 0.05$).

Acrosome integrity was higher in G13E41 than G8E24, G0E32, G0E54, G76E0, G19E57 and G0E76 at 0 h (Table 1, $p < 0.05$). Acrosome integrity was higher in G13E41 than G32E0, G0E32, G76E0 and G19E57 at 3 h ($p < 0.05$). Acrosome integrity was higher in

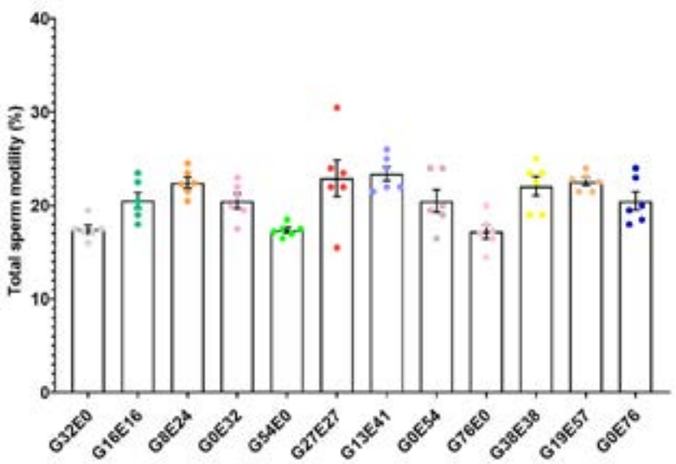

Figure 1
Effect of glycerol and erythritol on the total motility of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

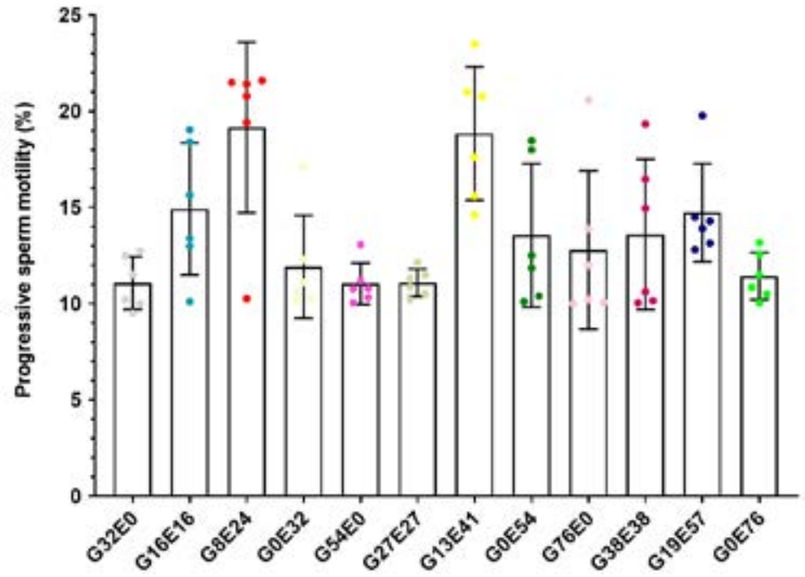

Figure 2
Effect of glycerol and erythritol on the progressive motility of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

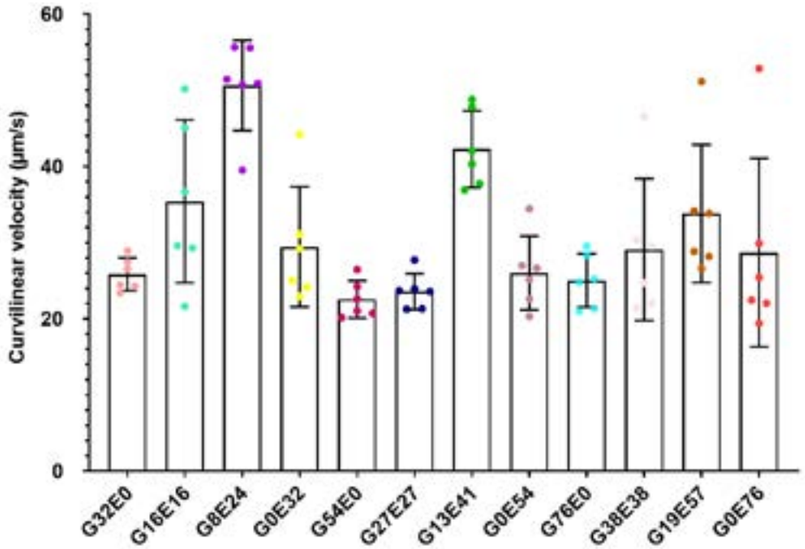

Figure 3
Effect of glycerol and erythritol on the curvilinear velocity of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

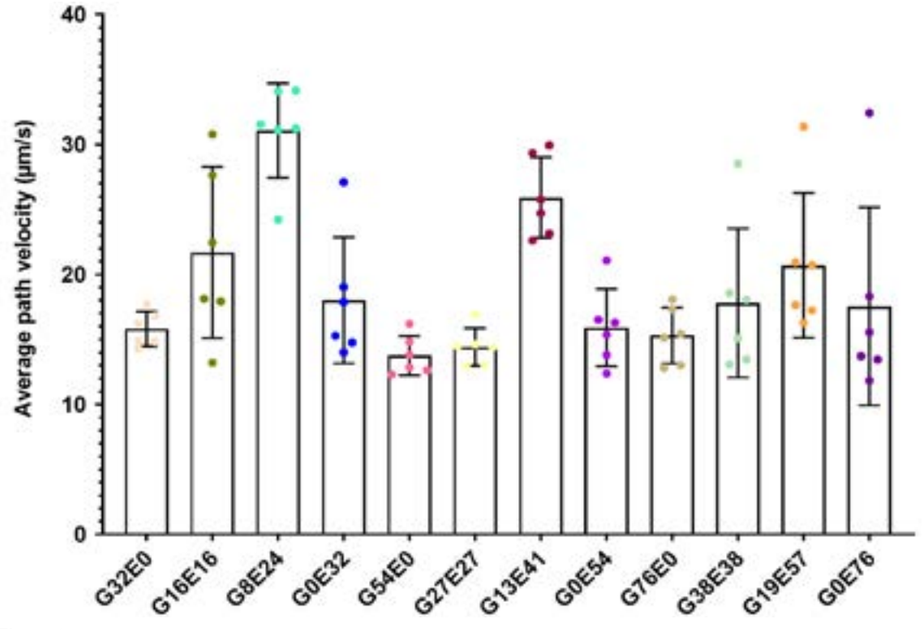

Figure 4
Effect of glycerol and erythritol on the average path velocity of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

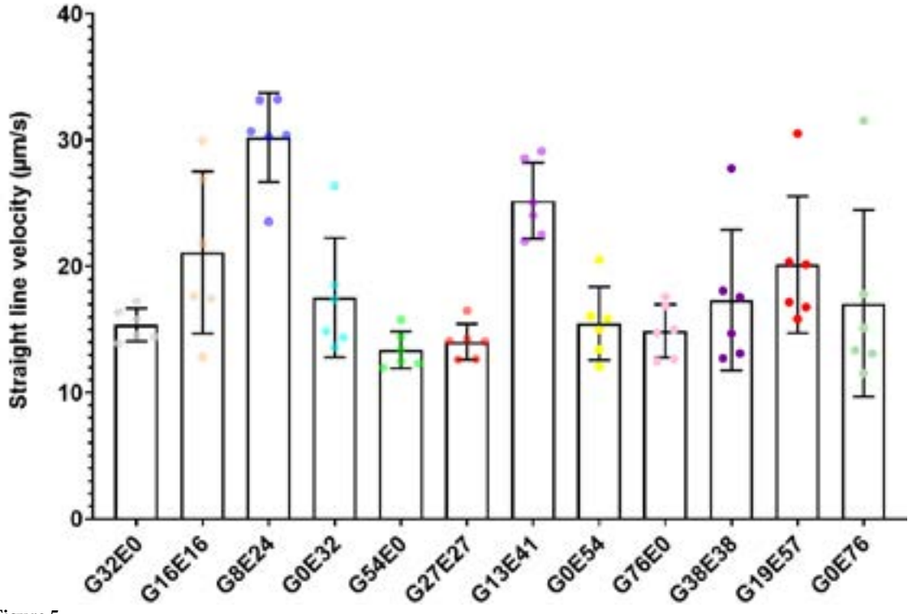

Figure 5
Effect of glycerol and erythritol on the straight line velocity of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

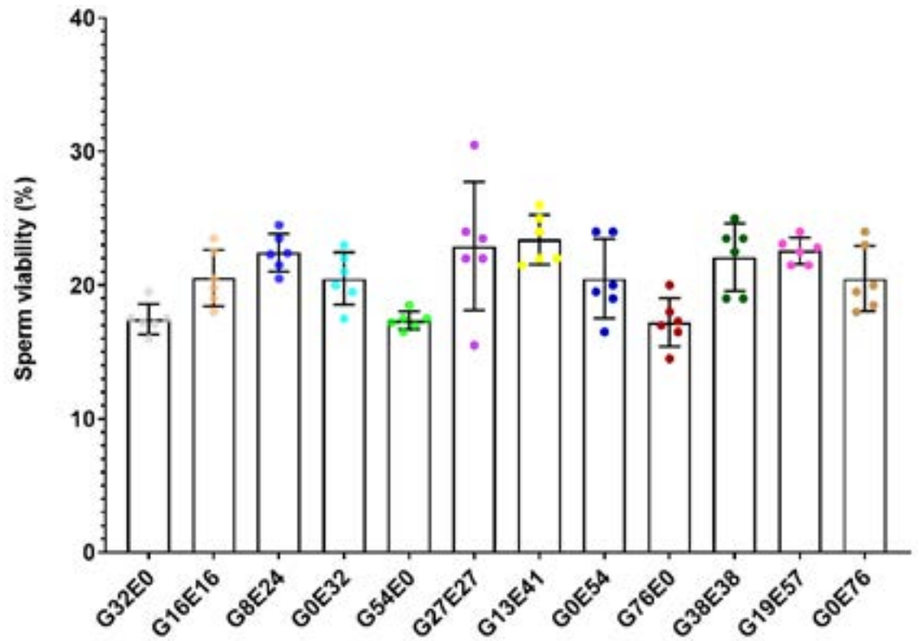

Figure 6
Effect of glycerol and erythritol on the viability of ram spermatozoa at 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

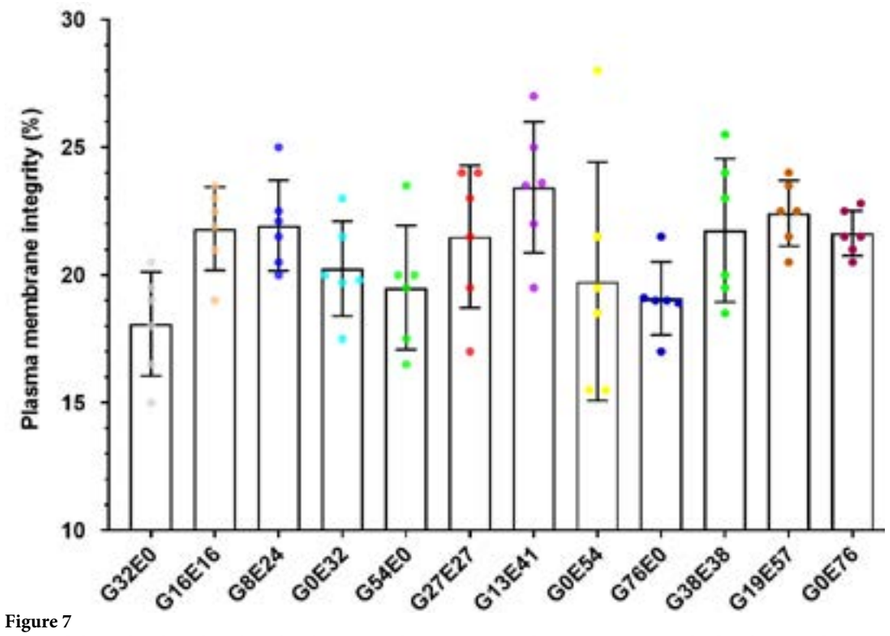

Figure 7
Effect of glycerol and erythritol on the plasma membrane integrity of ram spermatozoa 6 h after thawing. G32E0: 0.032 M glycerol, G16E16: 0.016 M glycerol + 0.016 M erythritol, G8E24: 0.008 M glycerol + 0.024 M erythritol, G0E32: 0.032 M erythritol, G54E0: 0.054 M glycerol, G27E27: 0.027 M glycerol + 0.027 M erythritol, G13E41: 0.013 M glycerol + 0.041 M erythritol, G0E54: 0.054 M erythritol, G76E0: 0.076 M glycerol, G38E38: 0.038 M glycerol + 0.038 M erythritol, G19E57: 0.019 M glycerol + 0.057 M erythritol, G0E76: 0.076 M erythritol.

Table 1
Effect of glycerol and erythritol on acrosome integrity at 0, 3 and 6 h after thawing.

Treatments*	Acrosome integrity (%)		
	0(h)	3 (h)	6 (h)
G32E0	30.25^{abc} ± 0.96	25.50^{b} ± 0.89	20.8^{b} ± 0.77
G16E16	31.75^{abc} ± 0.72	27.58^{ab} ± 1.14	20.17^{b} ± 1.35
G8E24	29.50^{bc} ± 0.65	26.83^{ab} ± 1.41	24.57^{ab} ± 1.03
G0E32	29.92^{b}c ± 1.39	25.33^{b} ± 0.99	21.8^{3b} ± 1.35
G54E0	31.25^{abc} ± 1.70	27.50^{ab} ± 1.64	20.58^{b} ± 0.7^{0}
G27E27	35.83^{ab} ± 1.66	30.75^{ab} ± 2.11	24.42^{ab} ± 1.04
G13E41	36.83^{a} ± 2.18	31.92^{a} ± 1.69	27.41^{a} ± 1.87
G0E54	28.75^{c} ± 0.82	26.58^{ab} ± 1.00	23.50^{ab} ± 1.10
G76E0	29.25^{bc} ± 1.70	25.08^{b} ± 0.72	20.58^{b} ± 0.70
G38E38	32.67^{abc} ± 1.77	28.25^{ab} ± 1.70	24.58^{ab} ± 0.82
G19E57	27.83^{c} ± 1.46	24.75^{b} ± 0.63	22.33^{ab} ± 0.84
G0E76	29.92b^{c} ± 1.15	27.25^{ab} ± 0.67	21.33^{b} ± 1.33

* G3E0: 0.032 M glycerol, G3E1/2: 0.016 M glycerol + 0.016 M erythritol, G3E3/4: 0.008 M glycerol + 0.024 M erythritol, G3E1: 0.032 M erythritol, G5E0: 0.054 M glycerol, G5E1/2: 0.027 M glycerol + 0.027 M erythritol, G5E3/4: 0.013 M glycerol + 0.041 M erythritol, G5E1: 0.054 M erythritol, G7E0: 0.076 M glycerol, G7E1/2: 0.038 M glycerol + 0.038 M erythritol, G7E3/4: 0.019 M glycerol + 0.057 M erythritol, G7E1: 0.076 M erythritol.
$^{a-c}$ Different letters show significant differences ($p < 0.05$).

G13E41 than G32E0 at 6 h ($p < 0.05$).

Discussion

The methods of freezing ram spermatozoa have not well been optimized yet. The physical stresses of freezing process induce irreversible damage to the sperm organelles and enzymatic activities, which have been associated with sperm functionality and fertility [12]. This may be minimized by the inclusion of the suitable cryoprotectant for ram semen.

Results of this study showed that there was no difference between G32E0 and G0E32, G54E0 and G0E54 and also G76E0 and G0E76 on the total and progressive sperm motility, viability, membrane integrity and acrosome integrity. Consequently, in the process of freezing of ram spermatozoa, a complete replacement of glycerol with erythritol is possible. Similarly, erythritol is used for freezing bull and boar spermatozoa, human erythrocytes and some animal embryos [9, 13, 14, 15, 16, 17]. It has been mentioned that the cryoprotective effect of the cell-penetrating polyols are enhanced along with the increase in their hydroxyl groups [9]. When polyols are used, these substances replace the water around the phospholipid head groups and the hydrogen bond between the hydroxyl group of polyol and the phospholipid phosphate groups is formed that protect the membrane against the damage of freezing procedure [18]. Both erythritol and glycerol penetrate the membrane cell, but the permeability of membrane to glycerol is higher than erythritol, and glycerol has one less hydroxyl group than erythritol [9, 18]. Erythritol was as effective as glycerol for protection against freezing-induced damage to ram spermatozoa, although, their number of hydroxyl groups and ability to pass through the cell membrane were different.

Results showed that sperm viability was higher in G8E24, G13E41, G27E27 and G19E57 than G32E0 and G76E0. Membrane integrity was higher in G13E41 than G32E0. Moreover, progressive sperm motility was highest in G8E24 at 0.032 M cryoprotectant. At 0.032 and 0.054 M cryoprotectant, the highest of the total sperm motility was observed in G8E24 and G13E41, respectively. Consequently, the mixture of erythritol and glycerol appears to provide protection to ram spermatozoa against the physical stress of freezing. Based on our knowledge, erythritol is free of side effects in regular use and also it is able to exert antioxidant activity in a cellular system [10, 11]. It has been indicated that erythritol was not mutagenic for bacterial cells and did not cause chromosomal damage to mammalian cells either *in vitro* or *in vivo* [19]. Moreover, it was illustrated that erythritol reduces the mitochondrial damage and increases the fertility of boar sperm after freezing-thawing [16]. On the other hand, glycerol is actively metabolised to formaldehyde, and high level of formaldehyde can destroy cell membranes [11]. Formaldehyde, highly reactive compound, is a strong inducer of apoptosis and lipid peroxidation [20]. Furthermore, it has been illustrated that removing glycerol via dialysis improve the fertility of frozen ram spermatozoa [5]. It was suggested that reducing the concentration of glycerol in the semen extender might be beneficial to the survival of spermatozoa when glycerol has been mixed with other cryoprotectants [21]. The combination of adonitol (up to 450 mM), as a low molecular weight polyol, and low levels of glycerol (1.5% v/v) improved the quality of pellet-frozen ram spermatozoa, whereas the high molecular weight polyols/glycerol combination had a detrimental effect [15]. Observations showed that the mixture of cryoprotective agents and saccharides provided better result than individual agents[22]. On the one hand, it was reported that if membrane integrity is supported by modifying the content of membrane cholesterol, the low concentration of glycerol (0.032 M) can protect ram spermatozoa during freezing [23]. Moreover, erythritol appears to stabilize membrane of bacteria [24]. According to the improvement of ram sperm quality caused by supplementation of semen extender with a low concentration of glycerol plus erythritol, therefore, the mixture of these polyols (at 0.032-0.054 M concentration) had a more protective effect on the frozen ram spermatozoa.

Our results showed that total sperm motility was higher in G8E24 than other treatments except for G13E41. Acrosome integrity was higher in G13E41 than G8E24 at 0 h, although, there was no difference between them at 3 and 6 h after thawing. At 6 h, acrosome integrity was higher in G13E41 than treatments containing only glycerol. Consequently, both the mixture of 0.013 M glycerol plus 0.041 M erythritol and 0.008 M glycerol plus 0.024 M erythritol improved longevity of frozen ram spermatozoa. Cryopreservation induces premature acrosome reactions [25]. Moreover, glycerol induces premature acrosome reaction and this lowered fertilizing capacity of the spermatozoa [5]. Furthermore, it was mentioned that glycerol had a detrimental effect on the acrosome integrity of ram spermatozoa [26]. Glycerol, as a formaldehydrogenic compound, may be considered as an exogenous source of formaldehyde [27]. The low concentration of formaldehyde increases intracellular calcium concentration in cultured hippocampal neurons via NMDARs and T-type Ca2+ channels [28]. Intracellular calcium is an important intracellular messenger and plays a key role in many physiological processes such as acrosome reaction [29]. Therefore, reducing the concentration of glycerol and replacing it with erythritol may improve the quality of the ram spermatozoa in the process of semen freezing.

In conclusion, our study demonstrates for the first time that ram spermatozoa are protected by erythritol against the physical stress of freezing. Moreover, the concentration of glycerol can be reduced by supplementation of semen extender with erythritol in semen cryopreservation. Furthermore, the quality of frozen-thawed ram spermatozoa can be improved by using the mixture of 0.013 M glycerol plus 0.041 M erythritol or 0.008 M glycerol plus 0.024 M erythritol.

Material and methods

Chemical reagents

The following chemicals and materials were used: meso-Erythritol (Sigma-Aldrich, St. Louis, MO, USA), Tris [hydroxymethyl] aminomethane, citric acid monohydrate, glucose, fructose and sodium citrate dihydrate, polyvinyl alcohol, glutaraldehyde, hoechst bisbenzimide 33258 (AppliChem GmbH, Darmstadt, Germany), potassium hydrogen phosphate, sodium hydrogen phosphate, sodium bicarbonate, ammonium sulfate, ammonium bicarbonate, sodium chloride, potassium chloride, methanol and glycerol 99.5% (Merck, Darmstadt, Germany), Alexa Fluor-488-PNA conjugate (Molecular Probes, Eugene, OR, USA).

Animals

This experiment was performed at University of Guilan, Faculty of Agricultural Sciences, Education Research and Practice Farm, South of Rasht (it is located at 37° 12′north latitude and 49° 39′east longitude). Four healthy mature Taleshi rams at age between 3 and 5 years were used. Rams were fed daily with a diet of 1300 g alfalfa hay, 620 g rice straw, 590 g barley and 100 g concentrates. Animals had free access to mineral supplement and fresh water. Animals cared for under experimental procedures and protocols approved by the Veterinary Organization of Iran.

Semen collection

Ejaculates (n=24) were collected from four rams by artificial vagina twice a week during the breeding season for six times. After ejaculation, the semen was diluted 1: 2 (v/v) with Tris diluents (300 mM tris [hydroxymethyl] aminomethane, 95 mM citric acid monohydrate, 28 mM D-glucose, 25 µg/mL gentamycin, pH 7.0) which containing 15% egg yolk. The samples were immersed in 33°C water and transferred to the laboratory by Styrofoam box within 10 min after collection [2].

Semen dilution, freezing and thawing

Upon reaching the laboratory, evaluation of samples was performed immediately. All diluted ejaculates were tested to possess an acceptable volume (> 0.5 mL), progressive motility (> 70%) and concentration (> 2.5 ×10^{9} sperm/mL).

In each session, the ejaculates were pooled, diluted up to 1.2×10^9 (cell/mL) by Tris-egg yolk (15%) and split into 12 equal parts. It was added to diluted semen 0.032 M, 0.054 M and 0.074 M glycerol and/or erythritol. The amount of 0.032 M glycerol (G32E0), 0.016 M glycerol and 0.016 M erythritol (G16E16), 0.008 M glycerol and 0.024 M erythritol (G8E24), 0.032 M erythritol (G0E32), 0.054 M glycerol (G54E0), 0.027 M glycerol and 0.027 M erythritol (G27E27), 0.013 M glycerol and 0.041 M erythritol (G13E41), 0.054 M erythritol (G0E54), 0.076 M glycerol (G76E0), 0.038 M glycerol and 0.038 M erythritol (G38E38), 0.019 M glycerol and 0.057 M erythritol (G19E57) and 0.076 M erythritol (G0E76) were added. Final concentration of sperm was 600×10^6 cells/mL. The diluted samples were packaged into 0.25 mL French straws, sealed with polyvinyl alcohol powder and cooled to 5°C over 2 h (0.25°C/min) and maintained at 5°C for 2 h. The straws were frozen in liquid nitrogen vapor, with the straws horizontally suspended 4 cm above the liquid nitrogen for 13 min, before being plunged into liquid nitrogen for storage [23]. Three straws for each replicate were thawed in a water bath at 37°C for 30s. Thawed semen incubated at 37°C for 6 h. Sperm motility, viability, functional membrane integrity and acrosome integrity were assessed immediately after thawing (0 h), subsequently after 3 h and 6 h post-thawing incubation.

Sperm assessment

The concentration of spermatozoa was determined by means of a Neubauer haemocytometer.

Total and progressive sperm motility were analyzed using a CASA system (Animal Version 6.51HFT CASA, Iran), digital colour camera (Samsung, Techwin, CO LTD, SCB-2000, Korea), phase-contrast microscope (GX Microscopes, Australia) equipped with heated stage set at 37°C. The sperm analyser was set-up as follows: frame rate – 50 Hz; minimum contrast – 50; low and high static-size gates – 0.53–4.45. Pre-warmed chambers slide (Sperm Processor, India) was loaded with 5 µL of a diluted sample at a concentration of 2×10^6 sperm/mL. Sperm motility parameters were recorded at 400× magnifications for 20 microscopic fields, at 37°C.

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane [2]. HOST was performed by incubating 5µL of semen with 50µL of a 100 mOsm hypo-osmotic solution (7.35 g sodium citrate dihydrate and 13.51 g fructose in 1000 mL distilled water) at 37°C for 30 min. One drop of the mixture was placed on a pre-warmed slide, covered with a cover slip and examined under a phase-contrast microscope (400×magnification). The sperm with swollen tails were considered intact. To assess the percentages of intact sperm, a total of 200 sperm were evaluated in at least five different microscopic fields.

Sperm viability was evaluated by fixed vital staining method [30]. Briefly, sample was mixed with an equal volume of a 2% glutaraldehyde solution (w/v) in phosphate-buffered saline (PBS), then it was mixed with an equal volume of 20 µg/mL bisbenzimide H33258. A smear was prepared after 10 min of incubation at room temperature. Two hundred spermatozoa per smear were evaluated in 3-7 different microscopic fields for each sample using an Olympus IX70, phase-contrast microscope (high-pressure mercury illuminator, UG1 excitation filter, U dichroic mirror, L420 barrier filter; Olympus, Tokyo, Japan). The procedure was performed by epifluorescence microscopy combined with bright-field illumination. Light intensity of the microscope was set at an optimum for visualization of both spermatozoa and fluorescence of H33258-labeled nuclei. Sperm showing partial or complete blue color was considered as dead, and sperm showing without color was considered to be alive.

Sperm acrosome integrity was estimated by staining with Alexa Fluor-488-PNA conjugate [2]. Briefly, sperm samples from each treatment were smeared on microscopic slides and air-dried. The samples were then fixed with methanol and kept at room temperature until staining procedure. For staining, ram sperm were incubated with 10 µg/mL Alexa Fluor-488-PNA in the darkness at 37°C for 30 min, washed with PBS and then analyzed under epifluorescence microscope (Olympus IX70) using an appropriate filter. In each sample, approximately 200 sperm were evaluated to determine the proportion of sperms with intact acrosome.

Statistical analyses

All data were analyzed by ANOVA followed by Tukey's test via the GLM procedure of SAS (version 9.1, SAS Institute Inc., 2002). The data of sperm motility, viability, function membrane integrity and acrosome integrity were analyzed by completely randomized design with 12 treatments in six replications. Differences were considered to be statistically significant at $p < 0.05$. Results were reported as means ± SE.

Author Contributions

M.A. performed the experiments. M.R.A.M. designed the research project and drafted the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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